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PRODUCTION OF LIPASE BY *PSEUDOMONAS FRAGI* IN A SYNTHETIC MEDIUM

Abstract

Alford, John A. (U.S. Department of Agriculture, Beltsville, Md.) and David A. Pierce. Production of lipase by *Pseudomonas fragi* in a synthetic medium. J. Bacteriol. **86**:24–29. 1963.—The growth and production of lipase by *Pseudomonas fragi* in 41 dehydrated media were determined. From a chemical analysis of the peptone medium which gave the maximal lipase production, numerous synthetic media were prepared. Good cell growth, but no lipase production, was obtained on a buffered ammonium sulfate-glucose medium. The addition of arginine, lysine, aspartic acid, and glutamic acid to this medium gave lipase production approaching that obtained in the peptone medium. Omission of the glucose had little effect on growth, but no lipase were produced. Sterilization of the glucose with either the amino acids or phosphate buffer had no effect on growth, but sharply reduced lipase production. Gas chromatographic analysis of the fatty acids liberated from lard, corn oil, and coconut oil indicated that medium composition had no effect on enzyme specificity. *P. fluorescens* produced very little lipase in any of the synthetic media examined.

Detailed studies on the mechanisms of synthesis of enzymes by microorganisms have been limited primarily to intracellular enzyme systems (Pardee, 1961; Halvorson, 1960). Among the extracellular enzymes, the effect of specific compounds on the production of proteinases has been the most actively investigated (Hartman, Zimmerman, and Rabin, 1957; Hagihara, 1960; Castañeda-Agulló, 1956; van der Zant, 1957). Microbial lipolysis has been studied frequently, but very little of this work has included studies with chemically defined media. Nashif and Nelson (1953) found that leucine, isoleucine, and valine caused an increase in lipase production by *Pseudomonas fragi*, although yields were considerably less than those in a peptone medium. They also found wide differences in lipase production with different protein digests. Cutchins, Doetsch, and Pelczar (1952) found that the production of lipase by *P. fluorescens* was increased when aspartic acid was used as the nitrogen source and olive oil as the carbon source, but their yields also were low. No lipase was produced when ammonium sulfate and glucose were the nitrogen and carbon sources. Ramakrishnan (1957) in studies on the in vitro synthesis of lipase by *Aspergillus niger* found that nine amino acids were probably involved in the synthesis of lipase protein.

Recent work in this laboratory on the activity of microbial lipases (Alford and Pierce, 1961; Alford et al., 1961) has indicated the desirability of more information about the factors influencing lipase synthesis. This paper is concerned with the development of a synthetic medium in which the production of lipase by *P. fragi* is similar to that produced in a peptone medium.

Materials and Methods

Source and cultivation of cultures. The *P. fragi* culture was obtained from W. C. Haynes, Northern Utilization Research and Development Division, U.S. Department of Agriculture, Peoria, Ill.; the *P. fluorescens* was ATCC 11251; and the unidentified *Pseudomonas* was isolated from crabmeat by M. A. Bernarde, Seafood Processing Laboratory, Crisfield, Md. During the early part of the investigation, the cultures were carried on 1% peptone agar buffered at pH 7.0 with 0.05 M phosphate. For the studies on growth in chemically defined media, they were carried on the basal medium (i.e., synthetic medium without amino acids) containing 1.5% agar. Cultures to serve as inocula were incubated at 20 C for 19 to 24 hr. Inocula were prepared by

washing the cells three times with sterile distilled water and adjusting to a constant turbidity.

Commercial media. The 41 media examined for their ability to support lipase production were prepared according to the manufacturer's directions when available. The casein hydrolysates and other proteins without specific recommendations for use were made up in a 1% concentration containing 0.05 M phosphate buffer and adjusted to pH 7.0. Sterilization was at 121 C for 10 min.

Production of lipase. Cultures were inoculated into 50 ml of medium in 300-ml Erlenmeyer flasks and incubated as stationary cultures at 20 C for 3 or 4 days. The cells were removed from the medium by centrifugation and the supernatant fluid was used as the enzyme.

Assay of lipase. Fat emulsions containing 20% of the fat or oil and 2% Astec 4135 (Associated Concentrates, Inc., Woodside, N.Y.) as the emulsifying agent were prepared by six passages through a Manton-Gaulin two-stage homogenizer at 4,000 psi. (Trade names are mentioned for identification, implying no endorsement.) The emulsion was kept under nitrogen during homogenization and stored under nitrogen at 1 to 3 C until used. The assays were carried out in Mojonnier fat-extraction flasks containing 2 ml of the fat emulsion, 2 ml of 0.25 M phosphate buffer (pH 7.0), and 4 ml of water. After equilibration at 35 C, 2 ml of enzyme were added and the flask was incubated for 30 min to 3 hr. Action of the enzyme was stopped by the addition of 1 ml of 1 N H_2SO_4. Alcohol (10 ml) was added, and the fat and fatty acids were extracted with two 20-ml portions of petroleum ether. The ether extract was titrated with 0.02 N alcoholic NaOH.

Gas chromatography. The types of fatty acids liberated were determined on a Beckman GC-2 gas chromatograph as described by Hornstein et al. (1960).

Results

A total of 41 different media were screened to determine their ability to support growth and lipase production. These included simple peptones, acid and pancreatic digests of casein, and several more complex media recommended for the growth of fastidious bacteria. Typical results are given in Table 1. Growth in all media was good, and maximal cell yields usually occurred in the media showing poor lipase production. Although the casein hydrolysates produced a wide range of activity, most of them gave good yields but none was quite equal to the 1% peptone medium.

TABLE 1. *Lipase production by Pseudomonas fragi grown in various commercial media*

Medium	Range of titratable acidity*
Peptones	
Difco	2.7–4.4
Case	3.7–4.5
Oxoid	0.5
Neopeptone (Difco)	0.8
Casein hydrolysates	0.1–4.5
Other media†	0.1–2.5

* Results expressed as ml of 0.02 N acid released from lard in 30 min.

† Thiotone, Trypticase Soy, and Phytone (all from BBL); proteose peptones, Gelatone, Protone, beef extract, and Tryptone (all from Difco).

From a chemical analysis of peptone furnished by Case Laboratories, Inc., Chicago, Ill., a synthetic medium was devised as shown in Table 2. The amino acids are divided into groups to facilitate subsequent elimination studies.

The stimulation of lipase production by glucose added to the synthetic medium is shown in Table 3. Since it was shown that an inorganic source of nitrogen could support fairly good growth, ammonium sulfate was included with all of the combinations of amino acids in subsequent trials so that available nitrogen would not be limiting. On the basis of the groupings shown in Table 2 and other subgroupings of these amino acids, it was found that only arginine, aspartic acid, glutamic acid, and lysine were required to give yields of lipase similar to that of the synthetic medium containing all 15 amino acids. Results with the best of these combinations, as well as with a leucine, isoleucine, and valine mixture similar to that used by Nashif and Nelson (1953), are shown in Table 4.

After determination of the amino acid requirements for good lipase production, the effect of the sodium and potassium concentrations in the phosphate buffer was determined (Table 5). In a similar manner, different concentrations of the four amino acids, ammonium sulfate, and glucose were tested to determine the most effective combinations for maximal yield. Although no sharp delineations were found, the following

TABLE 2. *Composition of original synthetic medium*

Substance	Amount	Substance	Amount
Amino acids (mg/100 ml)		Vitamins (μg/100 ml)	
Lysine	45	Biotin	1.3
Arginine	80	Niacin	35.0
Aspartic acid	60	Pyridoxine	2.5
Cystine	3	Riboflavine	5.0
Methionine	7	Thiamine	0.5
Glutamic acid	110	Mineral salts (mg/100 ml)	
Histidine	10	$MgSO_4 \cdot 7H_2O$	20
Proline	70	NaCl	1
Tyrosine	10	$FeSO_4 \cdot 7H_2O$	1
Tryptophan	2.5	$MnSO_4 \cdot 4H_2O$	1
Isoleucine	10	Adenine (mg/100 ml)	1
Leucine	35	Guanine (mg/100 ml)	1
Valine	30	Uracil (mg/100 ml)	1
Phenylalanine	25		
Threonine	15	Phosphate buffer (pH 7.0)	0.05 M

TABLE 3. *Effect of glucose on growth and lipase production by Pseudomonas fragi in a synthetic medium*

Medium	Growth*	Titratable acidity†
Case peptone medium (1%)	600	7.0
Synthetic medium (see Table 2)	400	0.1
+ 0.01% peptone	500	5.3
+ 0.1% glucose	500	6.2
+ 0.2% $(NH_4)_2SO_4$	400	0.2
+ 0.1% glucose and 0.2% $(NH_4)_2SO_4$	500	6.0
$(NH_4)_2SO_4$ (0.2%), 0.1% glucose, minerals, and buffer	300	0.1

* Reading on Klett-Summerson colorimeter.

† Results expressed as ml of 0.02 N acid released from lard in 1 hr.

medium consistently gave good yields and was adopted as the synthetic medium of choice: glutamic acid, 10^{-2} M; aspartic acid, 10^{-3} M; lysine, 10^{-3} M; arginine, 10^{-3} M; glucose, 5×10^{-2} M; $(NH_4)_2SO_4$, 2×10^{-2} M; phosphate buffer (pH 7.0; equal Na-K ratio), 10^{-1} M; mineral salts, same as in Table 2. It was found early in the work that sterilization of the glucose with the other ingredients affected lipase production without any apparent effect on growth (Table 6).

TABLE 4. *Effect of various amino acids, vitamins, and trace minerals on lipase production by Pseudomonas fragi*

Medium	Growth*	Titratable acidity†
Glucose (0.1%), 0.2% $(NH_4)_2SO_4$, 0.05 M phosphate buffer, minerals	250	0.2
without Fe, Mg, Mn, Na	Very slight	—
+ 15 amino acids	500	7.5
+ arginine, aspartic, glutamic, lysine	500	6.8
+ arginine, aspartic, glutamic, lysine, vitamins	500	6.6
+ arginine, aspartic, glutamic, lysine, peptone ash	500	6.8
+ arginine, aspartic, lysine	500	6.1
+ arginine, glutamic, lysine	500	5.0
+ aspartic, glutamic	500	4.8
+ leucine, isoleucine, valine	450	1.8

* Klett reading.

† Results expressed as ml of 0.02 N acid released from lard in 1 hr.

Lipase production by two additional pseudomonads was compared in three different media to determine whether the synthetic medium would satisfy their requirements for lipase production. *P. fluorescens* would not produce lipase in this medium (Table 7). Further experiments showed that no combination of the 15 amino acids, vitamins, and purines and pyrimidines would support much lipase production by this bacterium.

Work in our laboratory has shown that there are some differences in the specificities of lipases from different organisms. Since the yield of lipase is influenced by many factors, the specificity of the lipase produced in the synthetic medium was compared, by means of gas chromatography, with that produced in other media. Although slight variations were observed in the percentages of oleic and linoleic acids in individual fats, comparison of the three together indicates there was no apparent effect on specificity (Table 8).

TABLE 5. *Effect of sodium and potassium concentration on growth and lipase production in the synthetic medium*

Na	K	Growth*	Titratable acidity†
M	*M*		
0.00	0.05	500	2.7
0.01	0.04	500	2.7
0.02	0.03	500	3.9
0.03	0.02	500	4.4
0.04	0.01	300	0.3
0.05	0.00	150	0.2

* Klett reading.

† Results expressed as ml of 0.02 N acid released from lard in 30 min.

TABLE 6. *Effect of sterilization of glucose in presence of other ingredients on lipase production in synthetic medium*

Medium and sterilization treatment*	Titratable acidity†	pH after sterilization
All ingredients together	0.4	7.1
Glucose separately	7.4	7.2
Glucose and phosphate together	2.4	7.1
Glucose and amino acids together	1.0	7.2
Peptone (1%)-phosphate medium	9.0	7.2

* Sterilized at 115 C for 10 min; in all cases, the growth gave a Klett reading of 600.

† Results expressed as ml of 0.02 N acid released from lard in 1 hr.

TABLE 7. *Comparison of lipase production by three strains of Pseudomonas in three media*

Culture	Medium		
	Peptone	Casein digest	Synthetic
P. fragi	6.0*	5.2	5.0
P. fluorescens	1.5	3.5	0.5
Pseudomonas species	4.8	2.0	3.6

* Results expressed as ml of 0.02 N acid released from lard in 1 hr (3 hr for *P. fluorescens*).

TABLE 8. *Effect of growth medium on specificity of lipase produced by Pseudomonas fragi*

Growth medium	Per cent of total fatty acids								
	C_8	C_{10}	C_{12}	C_{14}	C_{16}	C_{18}	$C_{16}^{=}$	C_{18}^{-}	$C_{18}^{=}$
*Lard**									
Peptone					7	9	2	69	13
Casein digest					6	10	1	68	15
Synthetic					6	12	2	60	20
*Corn oil**									
Peptone					13	2		26	59
Casein digest					14	1		33	52
Synthetic					16	1		35	48
*Coconut oil**									
Peptone	10	16	46	19	8	tr		1	
Casein digest	8	14	50	17	10	tr		1	
Synthetic	8	15	50	16	10	tr		1	

* The lard, corn oil, and coconut oil were not part of the growth medium; they were the substrates for assaying the enzyme.

DISCUSSION

Lipolytic activity is commonly considered as a characteristic of taxonomic importance. The results obtained in this investigation point up the wide variability that may occur in lipase production by the same organism in different media. The failure of the *P. fluorescens* to produce appreciable lipase in the synthetic medium, as well as its production of more lipase on a casein digest than on peptone, is added evidence that a single medium is insufficient for the determination of lipolysis by different bacteria. This is particularly true if attempts are being made to differentiate degrees of lipolytic activity. Since *P. fluorescens* is similar to *P. fragi* in its pattern of lipolysis (Alford et al., 1961), the differences

in nutrient requirements for lipase synthesis probably are not caused by a basic difference in the two enzymes, but rather a difference in synthetic pathway. These data also support the conclusions shown by other workers (Goldman and Rayman, 1952; Nashif and Nelson, 1953) that lipase production is not a function of total cell growth.

The low level of lipase production by *P. fluorescens* in the presence of all amino acids and vitamins suggests that some di- or polypeptide may be required. Jayko and Lichstein (1959) showed that glycyl-L-asparagine caused considerable increase in the synthesis of lecithinase by *Clostridium perfringens*, and Davies (1962) found a cyclic dipeptide requirement for invertase synthesis by *Saccharomyces fragilis*.

Arginine, aspartic acid, glutamic acid, and lysine alone would account for over 90% of the total production of lipase when all 15 amino acids were included in the medium. Production obtained with isoleucine, leucine, and valine alone was similar to that found by Nashif and Nelson (1953), although the amount produced was considerably less than that obtained with the four amino acids mentioned above. Aspartic and glutamic acids are commonly involved in transamination reactions. Thus, the large stimulative effect by these two amino acids may be the result of their role in the synthesis of other amino acids rather than as a major constituent of the enzyme itself.

Contrary to the findings of Cutchins et al. (1952), vitamins had no apparent effect on lipase production. In their experiments, the amount of lipase produced in the presence of vitamins and ammonium sulfate was still less than 25% of that produced in nutrient broth. Therefore, it is possible that the apparent vitamin requirement they observed was related to an amino acid deficiency rather than to an absolute requirement for the vitamin.

It is well known that the ease with which a carbohydrate is fermented can be altered by sterilization with the other ingredients of the medium. It is of interest to note here, however, that the altered glucose has a definite effect on the synthesis of an enzyme unrelated to glucose metabolism, as reflected by cell growth. The effect of heating is not apparent when peptone is the substrate. This may be a protective effect by the polypeptides of the peptone, or it may be that some alternate pathway is involved.

Although the synthetic medium gave a slightly higher percentage of linoleic acid from lard than either the peptone or casein digest media, this trend was reversed in corn oil. Since these shifts tend to nullify each other, and since the error inherent in the method may be as much as 5 to 10%, it is doubtful that any effect on specificity occurred.

The lack of stimulation by the addition of peptone ash to the synthetic medium suggests that the only mineral requirements are Fe, Mg, Mn, Na, and K. The last two elements appear to be involved in lipase synthesis as well as cell growth. The apparent desirability of equimolar concentrations of these two for maximal lipase production is probably more related to minimal requirements for Na and K than to a fixed ratio of the two. Other experiments, in which the optimal concentrations of phosphate buffer in the synthetic medium were found to be 10^{-1} M, substantiate this.

It is possible, therefore, to utilize a synthetic medium for the production of from 80 to 90% of the amount of lipase produced by *P. fragi* in a medium containing peptone. Use of this medium will facilitate studies now underway on the purification of this enzyme.

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